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Influenza A virus-induced apoptosis is a multifactorial process: Exploiting reverse genetics to elucidate the role of influenza A virus proteins in virus-induced apoptosis☆

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Abstract

Three influenza viruses, A/Puerto Rico/8/34–A/England/939/69 clone 7a (H3N2), A/Fiji/15899/83 (H1N1), and A/Victoria/3/75 (H3N2), induce different levels of apoptosis in vitro at equal moi; Clone 7a > A/Victoria > A/Fiji. Previous studies have shown that several viral proteins from clone 7a and A/Fiji, including PB2, NA, NS1, M1, and M2, induce apoptosis when expressed individually fused to the herpes simplex virus tegument protein, VP22. However, this did not reflect viral protein–protein–RNA interactions known to occur within infected cells. To explore the role of viral proteins in apoptosis under infection conditions, recombinant viruses with single or triple gene exchanges were generated using A/Victoria or clone 7a as the background virus. Inserting the A/Fiji NS or PB2 gene into A/Victoria or clone 7a significantly reduced the level of apoptosis compared to the parent virus while clone 7a PA or NP genes increased apoptosis. Inserting A/Fiji NA or HA or clone 7a NS, M, NA, or HA genes individually into A/Victoria had no significant effect on apoptosis. Surprisingly, inserting the M, NA, and HA genes of A/Fiji together into clone 7a reduced apoptosis, whereas inserting clone 7a M, NA, and HA together into A/Fiji increased apoptosis. These results suggest that no single virus protein induces apoptosis and that the combination of genes required may be strain specific, highlighting the difficulty of predicting the virulence of new strains that arise in nature. No support for the view that apoptosis is essential for high virus yields was obtained as high virus yields were obtained with viruses that induced both high and low levels of apoptosis.

Keywords: Influenza A virus; Reverse genetics; Apoptosis; Virus proteins; Replication

Introduction

Influenza A virus induces apoptosis in a variety of cell types both in vitro and in vivo (Fesq et al., 1994; Govorkova et al., 1996; Hinshaw et al., 1994; Mori et al., 1995; Price et al., 1997; Takizawa et al., 1993). The reason why influenza virus induces apoptosis has been hotly debated. It was thought that apoptosis was primarily a host defense mechanism, limiting virus replication, and that influenza virus overcame this by rapid multiplication before apoptosis was induced (Kurokawa et al., 1999). However, there is now evidence that induction of apoptosis is essential for replication. Nuclear factor (NF)-κB dependent induction of TRAIL and Fas/FasL, and subsequent activation of caspase-3, all known to be involved in apoptosis induction (Fujimoto et al., 1998; Takizawa et al., 1995, 1999; Wada et al., 1995), are essential for virus propagation (Wurzer et al., 2003, 2004). Efficient viral mRNA synthesis has also been shown to correlate with apoptosis induction (Stray and Air, 2001).

☆ The nucleotide sequences of the PA and NP of clone 7a and A/Fiji have been submitted to the EMBL nucleotide sequence database and given the following accession numbers: clone 7a PA, AJ605763; A/Fiji PA, AJ605762; clone 7a NP, AJ628067; A/Fiji NP, AJ628066.

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Several viral proteins have been implicated in modulating apoptotic responses in infected cells. The dsRNA binding non-structural protein NS1 appears to down-regulate apoptosis (Morris et al., 2002; Zhirnov et al., 2002), although when expressed from a plasmid, in the absence of virus replication, this protein induces apoptosis (Morris et al., 2002; Schultz-Cherry et al., 2001). Neuraminidase (NA) activates transforming growth factor (TGF)-β, a known inducer of apoptosis in epithelial cells (Morris et al., 1999, 2002; Schultz-Cherry and Hinshaw, 1996). It is believed that NA removes sialic acid residues attached to carbohydrates on the latent (TGF)-β binding protein, which is complexed with pro-(TGF)-β. This allows the subsequent removal of the carbohydrate, a pre-determinant for the proteolytic cleavage of pro-(TGF)-β and release of the active molecule (Miyazono and Heldin, 1989). The newly discovered PB1-f2 induces apoptosis in vitro, in a cell-specific manner (Chen et al., 2001), by the formation of ion channels in the mitochondrial membrane (Chanturiya et al., 2004; Gibbs et al., 2003). PB1 and PB2 have also been implicated in the induction of apoptosis. Different levels of apoptosis induced in lymphocytes infected with either a virulent or non-virulent H5N1 influenza virus strain correlated with amino acid changes in PB1, PB2, and NA (Katz et al., 2000; Tumpey et al., 2000). The matrix protein, M1, interacts directly with, and may inhibit, caspase-8 (Timofeeva et al., 2001; Zhirnov et al., 1999). However, when expressed individually from a plasmid, both M1 and M2 induced apoptosis (Morris et al., 2002).

These conflicting results may be due to the experimental conditions used, e.g., expression of individual viral proteins in the absence of others or essential intermediates of viral replication such as dsRNA. Alternatively, as some viruses induce different levels of apoptosis when inoculated at equal moi (Mohsin et al., 2002; Price et al., 1997), viral proteins from different strains may differ in their ability to modulate the apoptotic response. Clearly, the role of each viral protein in influenza virus-induced apoptosis still needs to be resolved.

In this study, the RNA polymerase I reverse genetics system was employed to explore the role of each of the viral proteins in influenza virus-induced apoptosis. Recombinant viruses, with single or triple gene exchanges, were generated from 3 parent strains, A/Puerto Rico/8/34–A/England/939/69 clone 7a (H3N2), A/Fiji/15899/83 (H1N1) (A/Fiji), and A/Victoria/3/75 (H3N2) (A/Victoria). These three viruses induce different levels of apoptosis in vitro, clone 7a induces greater levels of apoptosis than A/Victoria which, in turn, induces greater levels of apoptosis than A/Fiji (Mohsin et al., 2002). These differences were exploited to investigate the role of each viral protein in different genetic backgrounds. We show that the level of apoptosis induced by each virus strain cannot be predicted by its genetic configuration as the viral gene exchanged and the background into which it is inserted influence the level of apoptosis induced. In addition, there is no evidence to support the view that replication is related to the level of apoptosis induced.

Results

Generation of recombinant viruses

The viruses generated using the twelve plasmid reverse genetics system are described in Table 1. The genotype of each virus was confirmed by RT-PCR and sequence analysis. Three recombinant viruses with single gene exchanges could not be generated. Five attempts to generate A/Victoria and clone 7a recombinant viruses containing the A/Fiji M gene and six attempts to generate A/Victoria recombinant viruses containing the A/Fiji PB1 gene were unsuccessful. However, the same pPolFM and pPolFPB1 plasmid preparations were used to generate rFiji, indicating that the failure was not due to the construction or preparation of these plasmids and that introducing A/Fiji M or PB1 into A/Victoria may result in production of non-viable virus. This may be due to the inserted gene product being incompatible with one or more of the background virus gene products.

Table 1
Recombinant viruses generated using the 12 plasmid reverse genetics system

Background virus	RNA segment(s) introduced	Recombinant virus
A/Victoria		rVictoria
	Fiji NS	rVicFNS
	Fiji NA	rVicFNA
	Fiji NP	rVicFNP
	Fiji HA	rVicFHA
	Fiji PA	rVicFPA
	Fiji PB2	rVicFPB2
	Fiji PA, PB2, PB1	rVicFpoly
	7a NS	rVic7aNS
	7a M	rVic7aM
	7a NA	rVic7aNA
	7a NP	rVic7aNP
	7a HA	rVic7aHA
	7a PA	rVicPA
	7a PB2	rVic7aPB2
	7a PB1	rVic7aPB1
	7a PA, PB2, PB1	rVic7apoly
clone 7a		r7a
	Fiji NS	r7aFNS
	Fiji NA	r7aFNA
	Fiji NP	r7aFNP
	Fiji HA	r7aFHA
	Fiji PA	r7aFPA
	Fiji PB2	r7aFPB2
	Fiji PB1	r7aFPB1
	Fiji M, NA, HA	r7aFM:NA:HA
A/Fiji		rFiji
	7a NS	rFiji7aNS
	7a M	rFiji7aM
	7a M, NA, HA	rFiji7aM:NA:HA

Calculation of percentage total apoptotic cell death (apoptosis)

We have previously shown that clone 7a and A/Fiji induce apoptosis rather than necrosis in MDCK cells using morphological analysis, DNA laddering, and TUNEL (Brydon et al., 2003; Price et al., 1997). We have also shown that calculating total apoptotic cell death from percentage cytotoxicity and morphological apoptosis is essential for comparing viruses (Mohsin et al., 2002). Surprisingly, the level of infection induced by different viruses was not uniform despite the use of a high moi (4 $TCID_{50}$/cell). For example, A/Fiji induced an infection level of 95–100%, A/Victoria of 50–75%, and clone 7a of 95–100%. This was also evident with the recombinant viruses. Since the total level of infection induced by different viruses was reproducibly different, it was not appropriate to compare the total number of apoptotic cells induced by different viruses without reference to the total number of infected cells. This is meaningful as we have never observed in this system an apoptotic cell that was not infected.

Thus, to compare viruses, the number of apoptotic cells is expressed as a percentage of the total number of infected cells.

Total apoptotic cell death induced by the parent and recombinant viruses

A/Victoria, clone 7a, and A/Fiji differ in the level of apoptosis induced at equal moi (5 50% egg infectious doses/cell); clone 7a > A/Victoria > A/Fiji (Mohsin et al., 2002). During this study, cells were infected with an moi of either 2 or 4 $TCID_{50}$/cell. To confirm that the differential induction of apoptosis is still observed at equal $TCID_{50}$, MDCK cells were infected with 4 $TCID_{50}$/cell of clone 7a, rVictoria, or A/Fiji, and the level of apoptosis determined 24 h after infection. In 2 experiments, the levels of total apoptotic cell death were 58.2% (SD 7.9) and 74.4% (SD 4.3) for clone 7a, 28.8% (SD 4.0) and 39.4% (SD 6.0) for A/Victoria, and 13.7% (SD 3.7) and 9.6% (SD 2.2) for A/Fiji. The level of total apoptotic cell death varied between experiments, but in each experiment the difference between the three viruses was significant ($P < 0.05$). The levels of total apoptotic cell death induced by the recombinant viruses, r7a and rFiji, were not significantly different ($P > 0.05$) from that of the parent viruses, clone 7a and A/Fiji, respectively; 57.3% (SD 1.6) for r7a compared to 56.7% (SD 5.0) for clone 7a and 7.1% (SD 0.8) for rA/Fiji compared to 7.3% (SD 2.1) for A/Fiji.

Apoptosis induced by recombinant viruses containing A/Fiji or clone 7a NS segments

MDCK cells were infected with 4 $TCID_{50}$/cell of clone 7a, rVictoria, A/Fiji, rVicFNS, r7aFNS, rVic7aNS, or rFiji7aNS, and the level of apoptosis determined 24 h after infection. A typical experiment is shown in Fig. 1. rVicFNS induced less apoptosis than rVictoria (Fig. 1) in all 6 experiments. In 5 of these, this reduction was significant ($P < 0.05$). The level of apoptosis induced by rVicFNS was similar to that observed for A/Fiji. Inserting A/Fiji NS gene into clone 7a also significantly reduced ($P < 0.01$) the level of apoptosis induced (Fig. 1) with respect to clone 7a in all 6 experiments. Again the level of apoptosis induced by r7aFNS was not significantly different ($P > 0.05$) to that of A/Fiji.

In contrast, introducing clone 7a NS into rVictoria did not significantly affect ($P > 0.05$) the level of apoptosis induced in any of 6 experiments (Fig. 1), although in 5 of these the level induced by rVic7aNS was higher than that of rVictoria. Due to the interesting results obtained for viruses containing A/Fiji NS, rFiji7aNS virus was generated for

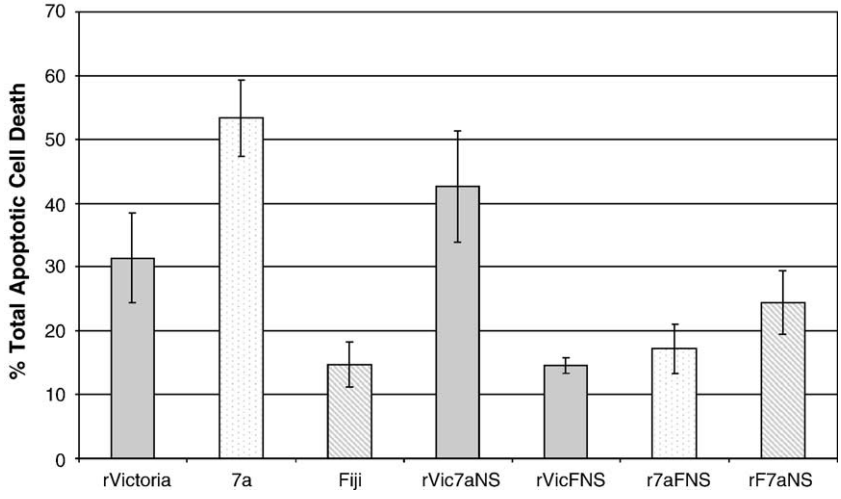

Fig. 1. Total apoptotic cell death induced by viruses with exchanged NS genes. MDCK (NBL-2) cells were infected with 4 $TCID_{50}$/cell of rVictoria, clone 7a, A/Fiji, rVic7aNS, rVicFNS, r7aFNS, or rFiji7aNS. Columns represent the mean percentage of total apoptotic cell death of 3 replicate samples (±SD) 24 h pi.

comparison. Inserting clone 7a NS gene into A/Fiji increased the total apoptotic cell death induced compared to A/Fiji (Fig. 1). In 2 out of 3 experiments, this increase was significant ($P < 0.05$). However, the level of apoptosis was significantly lower ($P < 0.01$) than that induced by clone 7a.

Apoptosis induced by recombinant viruses with exchanged NA and HA genes

MDCK cells were infected with either 4 $TCID_{50}$/cell of clone 7a, rVictoria, A/Fiji, rVicFNA, r7aFNA, or rVic7aNA, or 2 $TCID_{50}$/cell of clone 7a, rVictoria, A/Fiji, rVicFHA, r7aFHA, or rVic7aHA, and the level of apoptosis determined 24 h after infection. Different inocula were necessary for comparison as some viruses did not grow sufficiently well to allow comparison at the standard inoculum of 4 $TCID_{50}$/cell. Five to six experiments were performed for each virus and 3 representative experiments are shown in Table 2. Inserting clone 7a or A/Fiji NA gene into A/Victoria had no significant effect ($P > 0.05$) on the level of apoptosis induced compared to rVictoria in 6 and 5 out of 6 experiments, respectively (Table 2a). Similarly, r7aFNA did not induce a significantly different ($P > 0.05$) level of apoptosis compared to clone 7a (Table 2a).

The levels of apoptosis induced by rVic7aHA and r7aFHA were not significantly different ($P > 0.05$) from those induced by rVictoria and clone 7a, respectively, in 3 out of 5 experiments (Table 2b). Although not significant, in most experiments, the levels of apoptosis induced by both rVicFHA and r7aFHA were lower than that of the respective parent virus. The level of apoptosis induced by rVic7aHA was also not significantly different ($P > 0.05$) from that induced by rVictoria in 4 out of 5 experiments (Table 2b). However, no overall trend was observed for this virus.

NA activity and binding affinity of recombinant viruses with exchanged NA or HA genes

To maintain the appropriate balance between attachment/entry and release/spread, the NA and HA evolve together;

Table 2
Total apoptotic cell death, NA activity, and HI titer of recombinant viruses with exchanged (a) NA, (b) HA, and (c) M, NA, and HA

Virus	Percent total apoptotic cell death[a]			NA activity based on number of particles [μM NANA released over 18h] (SD)[b]	NA activity based on $4TCID_{50}$ [μM NANA released over 18h] (SD)[c]	HI titer[d]
	Experiment 1 (SD)	Experiment 2 (SD)	Experiment 3 (SD)			
a.						
rVictoria	28.2 (4.0)	38.0 (3.4)	50.4 (14.1)	0.21 (0.02)	0.05 (0.0)	32–64
Clone 7a	58.2 (7.9)	88.9 (1.6)	59.3 (6.5)	0.74 (0.12)	1.18 (0.23)	256–512
A/Fiji	13.7 (3.7)	19.0 (2.4)	16.2 (2.5)	0.22 (0.0)	1.05 (0.13)	8–16
rVic7aNA	20.9 (1.3)	38.3 (11.7)	48.4 (8.9)	0.73 (0.19)	0.23 (0.01)	64–128
rVicFNA	38.5 (14.4)	53.2 (18.3)	39.1 (8.5)	0.21 (0.17)	0.35 (0.09)	32
r7aFNA	53.1 (12.4)	86.1 (19.0)	ND	0.51 (0.04)	0.09 (0.0)	128–256
b.						
rVictoria	24.3 (2.4)	24.6 (3.1)	24.1 (3.7)	0.21 (0.02)		32–64
Clone 7a	72.6 (5.1)	45.5 (2.0)	70.7 (8.8)	0.74 (0.12)		512
A/Fiji 6.4	6.4 (2.3)	16.7 (6.1)	24.8 (4.9)	0.22 (0.0)		4–8
rVic7aHA	23.8 (4.0)	25.0 (3.3)	60.7 (18.2)	0.31 (0.01)		128
rVicFHA	19.5 (4.3)	14.6 (3.2)*	16.1 (5.3)	0.16 (0.01)		8
r7aFHA	43.7 (1.1)**	37.5 (9.8)	63.4 (7.3)	0.75 (0.13)		4–8
c.						
Clone 7a	52.8 (2.5)	74.4 (4.3)	80.7 (4.3)			
A/Fiji	5.3 (1.5)	9.6 (2.2)	6.3 (5.6)			
rFiji7aM:NA:HA	25.3 (2.1)**	57.8 (7.5)**	21.6 (4.8)*			
r7aFM:NA:HA	8.0 (1.4)**	12.1 (3.8)**	13.0 (6.5)**			

[a] Total apoptotic cell death was calculated from the level of cytotoxicity (the number of cells that have entered late apoptosis and lysed) and the level of morphological apoptosis (those cells that remain on the coverslip and have characteristic nuclear fragmentation as determined by propidium iodide staining). Standard deviations were calculated from 3 replicate wells ($n = 3$).

[b] NA activity measured using the standard WHO assay with fetuin as the substrate and is expressed as μM *N*-acetyl neuraminic acid (NANA) released over 18-h incubation period. All viruses were diluted to 80 HA units/ml for assay. The results show the means (±SD) of 2 replicates in a single experiment but are representative of 3 similar experiments.

[c] NA activity measured as above but all viruses diluted to the equivalent of 4 $TCID_{50}$/cell. The results show the means (±SD) of 2 replicates in a single experiment but are representative of 3 similar experiments.

[d] HI titer expressed as the reciprocal of the highest dilution of horse serum (α-macroglobulin) that still caused complete inhibition of hemagglutination.

* Significantly different from parent virus ($P < 0.05$).

** Significantly different from parent virus ($P < 0.01$).

viruses with strong affinity for their receptors require a highly active NA (Mitnaul et al., 2000). To ensure that the observed effects were due to the exchanged gene and not due to mutations in the HA arising from changing the NA, or from mutations in the exchanged NA due to the background HA and vice versa, the NA activities and binding affinity of the HA were determined. The NA activities of rVictoria, clone 7a, and A/Fiji, based on equal number of particles, showed that clone 7a > A/Fiji = rVictoria (Table 2a). The NA activity of the recombinant viruses, rVic7aNA and rVicFNA, based on equal number of particles, mirrored that of the parent from which the NA gene was derived (Table 2a). However, the NA activity of r7aFNA, based on equal number of particles, was significantly higher than that of A/Fiji ($P < 0.05$), although it was significantly lower ($P < 0.05$) than that of clone 7a. This difference in NA activity is not due to sequence changes in the NA gene per se as full-length sequence analysis of segment 6 from r7aFNA revealed 100% identity to segment 6 of A/Fiji.

The NA activities of rVic7aNA and rVicFNA at equal infectivity (4 $TCID_{50}$/cell, used in the apoptosis assays) were significantly higher ($P < 0.05$) than that of rVictoria (Table 2a) but significantly lower than that of clone 7a and A/Fiji. The NA activity of r7aFNA at equal infectivity was also significantly lower compared to clone 7a and A/Fiji. The NA activities of clone 7a and A/Fiji, at equal infectivity, although significantly higher ($P > 0.05$) than that of the recombinant viruses rVictoria, rVic7aNA, rVicFNA, and r7aFNA, were not significantly different ($P > 0.05$) from each other (Table 2a).

The differences in NA activities for the same virus calculated from particle numbers and $TCID_{50}$ probably reflect the variation (±2-fold) in HA estimations for the viruses. Nevertheless, they do show that the NA activities of the recombinant viruses were similar to those of the parent from which the NA was derived.

The NA activities of the recombinant viruses, rVic7aHA, rVicFHA, and r7aFHA, based on equal number of particles, were similar to the parent from which the NA gene was derived (Table 2b).

The binding affinity of the recombinant virus' HAs was determined, as judged by binding to erythrocytes in the presence of α-2 macroglobulin, the α-2,6 receptor inhibitor in horse serum (Mohsin et al., 2002). The binding affinities of rVic7aNA, rVicFNA, r7aFHA, rVicFHA, rVic7aHA, and r7aFHA were similar to the parent virus from which the HA was derived (Table 2b).

Apoptosis induced by recombinant viruses with exchanged M gene

As previously described, neither an A/Victoria nor a clone 7a recombinant virus containing A/Fiji M gene could be generated. When used to infect MDCK cells at a moi of 4 $TCID_{50}$/cell, both rVic7aM and rFiji7aM induced a level of apoptosis that was not significantly different ($P > 0.05$) from rVictoria [16.4 (SD 0.9) for rVic7aM compared to 24.0 (SD 3.6) for rVictoria] or A/Fiji, respectively [5.3 (SD 1.6) for rFiji7aM compared to 2.3 (SD 0.5) for A/Fiji].

Apoptosis inducing ability of recombinant viruses with exchanged HA, NA, and M genes

The above results suggested that generating a recombinant clone 7a virus containing A/Fiji M, NA, and HA genes (Table 1) or a recombinant A/Fiji virus containing clone 7a M, NA, and HA genes (Table 1) should still behave like clone 7a and A/Fiji, respectively. However, rFiji7aM/NA/HA induced significantly more apoptosis than A/Fiji, whereas r7aFM/NA/HA induced significantly less apoptosis than clone 7a in all 3 experiments (Table 2c). These results contradict those obtained for recombinant viruses with single gene exchanges, possibly indicating that apoptosis induction may depend on viral protein–protein interactions.

Apoptosis induced by recombinant viruses with exchanged NP genes

MDCK cells were infected with 2 $TCID_{50}$/cell of clone 7a, rVictoria, A/Fiji, rVicFNP, r7aFNP, or rVic7aNP, and the level of apoptosis determined 24 h later. Three to five experiments were performed for each virus and a typical experiment is shown in Fig. 2. rVicFNP induced a significantly higher ($P < 0.05$) level of apoptosis than rVictoria in all 5 experiments (Fig. 2). In contrast, r7aFNP induced a significantly ($P < 0.05$) lower level of apoptosis than clone 7a in 3 out of 4 experiments (Fig. 2). The level of apoptosis induced by rVic7aNP was also significantly greater ($P < 0.05$) than that induced by rVictoria in 3 out of 5 experiments. While not always significant, in all 5 experiments, the level of total apoptotic cell death induced by rVic7aNP was higher than that induced by rVictoria.

Apoptosis induced by recombinant viruses with exchanged PA, PB2, and PB1 genes

MDCK cells were infected with 4 $TCID_{50}$/cell of clone 7a, rVictoria, A/Fiji, rVicFPB2, r7aFPB2 rVic7aPB2, rVicFPA, r7aFPA, rVic7aPA, or rVic7aPB1. Inserting A/Fiji PA gene into A/Victoria had no significant effect ($P > 0.05$) on the level of apoptosis induced 24 h pi compared to rVictoria (Fig. 3), although the level of apoptosis induced was higher than that induced by rVictoria in 4 out of 5 of these experiments. In contrast, r7aFPA reproducibly induced less apoptosis than clone 7a, but again this reduction was only significant in 1 out of 3 experiments. rVic7aPA induced a significantly higher level ($P < 0.01$) of apoptosis than rVictoria in 4 out of 5 experiments (Fig. 3).

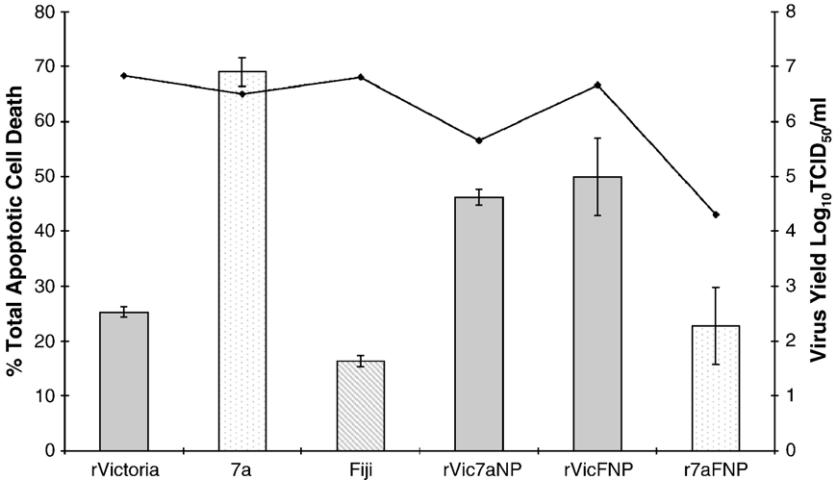

Fig. 2. Total apoptotic cell death induced and virus yields generated by viruses with exchanged NP genes. MDCK (NBL-2) cells were infected with 2 $TCID_{50}$/cell of rVictoria, clone 7a, A/Fiji, rVic7aNP, rVicFNP, or r7aFNP. Columns represent the mean percentage of total apoptotic cell death of 3 replicate samples (±SD) 24 h pi; data points represent virus yields in log10 $TCID_{50}$/ml.

rVicFPB2 induced less apoptosis than rVictoria (Fig. 4) in 4 out of 5 experiments; in 3 of these, this reduction was significant ($P < 0.05$). Similarly, 7aFPB2 induced significantly less ($P < 0.05$) apoptosis than clone 7a in 4 out of 5 experiments. However, the level of apoptosis induced by rVic7aPB2 was not significantly different from that induced by rVictoria.

As previously described, rVicFPB1 could not be generated. Interestingly, r7aFPB1 also grew poorly and only reached sufficient titers to be inoculated at an moi of 0.1 $TCID_{50}$/cell. A comparison with clone 7a at this moi showed that r7aFPB1 produced less apoptosis than clone 7a in 3 out of 3 experiments (typically 20.8% (SD 3.5) for r7aFPB1 compared to 68.4% (SD 7.9) for clone 7a). In 2 of these experiments, the reduction in apoptosis was significant ($P < 0.05$). The level of apoptosis induced by 4 $TCID_{50}$/cell rVic7aPB1 was not significantly different from rVictoria [51.94% (SD 10.7) for rVic7aPB1 compared to 46.9% (SD 5.3) for rVictoria] in any of the 3 experiments.

Apoptosis induced by A/Victoria recombinant viruses containing either clone 7a or A/Fiji PB2, PB1, and PA genes

To investigate the polymerase proteins further, recombinant viruses were generated which contained A/Fiji PB1, PB2, and PA (rVicFpoly) or clone 7a PB1, PB2, and PA gene (rVic7apoly) segments in an A/Victoria background (Table 1). rVicFpoly induced a level of apoptosis not significantly different to that induced by rVictoria (Fig. 5) in any of 3 experiments. In contrast, rVic7apoly induced more apoptosis than rVictoria in 3 experiments (Fig. 5) and, in 2 of these, this increase was significant ($P < 0.05$).

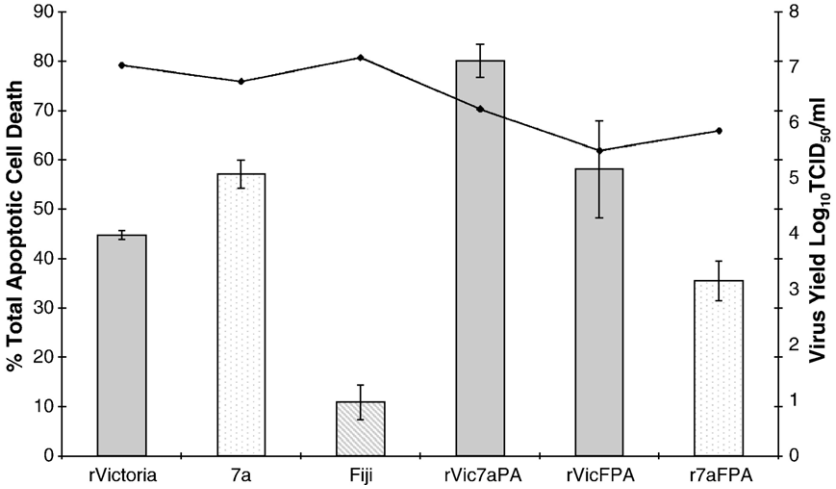

Fig. 3. Total apoptotic cell death induced and virus yields generated by viruses with exchanged PA genes. MDCK (NBL-2) cells were infected with 4 $TCID_{50}$/cell rVictoria, clone 7a, A/Fiji, rVic7aPA, rVicFPA, or r7aFPA. Columns represent the mean percentage of total apoptotic cell death of 3 replicate samples (±SD) 24 h pi; data points represent virus yields in log10 $TCID_{50}$/ml.

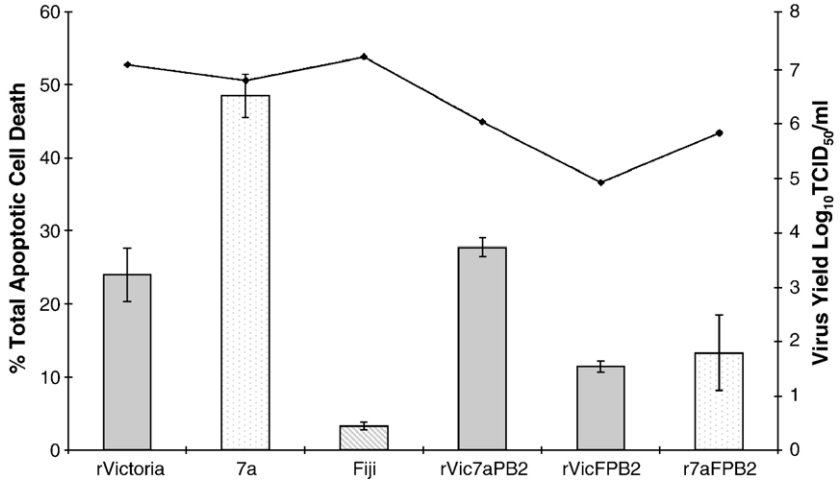

Fig. 4. Total apoptotic cell death induced and virus yields generated by viruses with exchanged PB2 genes. MDCK (NBL-2) cells were infected with 4 $TCID_{50}$/cell of rVictoria, clone 7a, A/Fiji, rVic7aPB2, rVicFPB2, or r7aFPB2. Columns represent the mean percentage of total apoptotic cell death of 3 replicate samples (±SD) 24 h pi; data points represent virus yields in log10 $TCID_{50}$/ml.

Virus titers generated by recombinant viruses with exchanged NP or polymerase proteins

As the polymerase proteins and NP are all involved in influenza virus replication, exchanging these genes might affect the rate and/or efficiency of replication. Therefore, virus yields were determined 24 h after infection. A/Fiji consistently induced a significantly lower level of apoptosis than clone 7a and rVictoria but produced similarly high viral yields (Figs. 2–5). Generally, recombinant viruses replicated less well than their parental viruses, but this appears to be independent of the level of apoptosis induced (Figs. 2–5). For example, rVic7aPA, rVicFPA, and r7aFPA virus yields were 10- to 50-fold lower than their respective parental viruses but whereas r7aFPA produced lower levels of apoptosis than clone 7a, both rVic7aPA and rVicFPA induced higher levels of apoptosis than rVictoria (Fig. 3). In contrast, the low yields of rVic7aPB2 and rVicFPB2 correlated with an increased level of apoptosis for the former and a decrease for the latter (Fig. 4). Similarly, the increased levels of apoptosis induced by rVic7aNP and rVicFNP were not associated with a change in virus yields (Fig. 2).

Discussion

Previous investigations of the role of individual viral proteins in influenza virus-induced apoptosis have relied on their expression in a range of mammalian cells or use of mutant, gene-knockout viruses. However, these approaches have problems in that expression of individual viral proteins ignores viral protein–protein–RNA interactions known to occur during infection. Here we have examined the role of the viral proteins during infection by exploiting the twelve

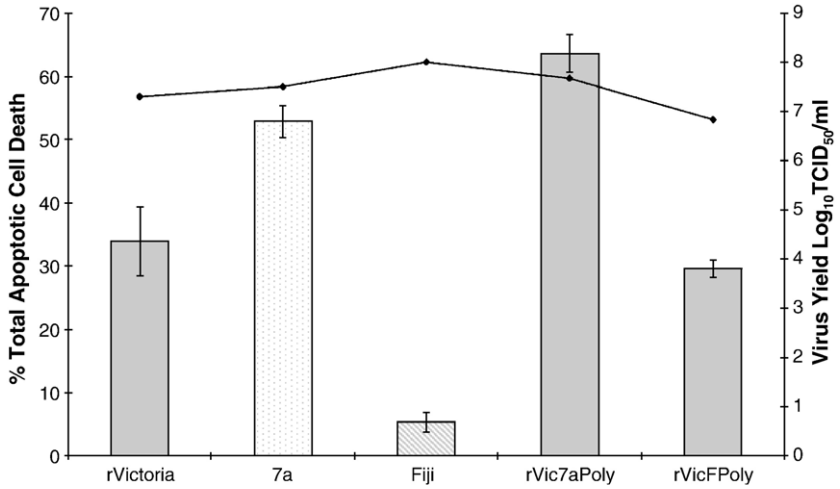

Fig. 5. Total apoptotic cell death induced and virus yields generated by viruses with exchanged PA, PB2, and PB1 genes. MDCK (NBL-2) cells were infected with 4$TCID_{50}$/cell of rVictoria, clone 7a, A/Fiji, rVic7aPoly, or rVicFpoly. Columns represent the mean percentage of total apoptotic cell death of 3 replicate samples (±SD) 24 h pi; data points represent virus yields in log10 $TCID_{50}$/ml.

plasmid reverse genetics system to generate a panel of viruses with single or triple gene exchanges.

The NS gene from A/Fiji caused a significant decrease in the level of total apoptotic cell death when inserted into A/Victoria or clone 7a (Fig. 1). This is the first time that any influenza virus gene has been shown to have a role in the differences observed in the apoptotic response between viruses. Segment 8 encodes 2 products, NS1 and nuclear export protein (NEP, NS2), but it is more likely that NS1 mediates the effect as apoptosis increased in cells infected with a NS1 deletion mutant (Zhirnov et al., 2002) and NS1 expressed from a plasmid reduced dsRNA-induced apoptosis (Morris et al., 2002). This is also supported by the observation that at the amino acid level A/Fiji NS1 is only 91% and 86% identical to that from A/Victoria and clone 7a, respectively, whereas A/Fiji NEP is 97% and 99% identical to that from A/Victoria and clone 7a, respectively (Fig. 6). However, all the amino acids known to be directly involved in the multiple functions of NS1 and NEP are conserved for all 3 viruses. NS1 may act directly by binding to PKR, an apoptosis mediator (Tan and Katze, 1998), sequestering dsRNA (Hatada and Fukuda, 1992) or inhibiting the c-Jun N-terminal kinase (JNK)/AP1 stress activated transcription pathway (Ludwig et al., 2002) known to be involved in the induction of apoptosis by equine influenza virus (Lin et al., 2001). Alternatively, NS1 may act indirectly by inhibiting late viral gene expression (Enami and Enami, 2000; Falcon et al., 2004) or by controlling the switch from transcription to replication (Falcon et al., 2004), which could affect both mRNA and dsRNA levels. However, a role for NEP cannot be excluded as it exports progeny ribonucleoprotein complexes out of the nucleus via its interaction with matrix protein 1 (M1) and nucleoporins (O'Neill et al., 1998). It also down-regulates the synthesis of vRNA, cRNA, and mRNA in a dose-dependent manner (Bullido et al., 2001). This could cause a reduction in either the dsRNA concentration within infected cells or the level of a viral-apoptosis-inducing protein.

We and others have reported that NA is involved in the induction of apoptosis (Morris et al., 1999; Schultz-Cherry and Hinshaw, 1996). Here, however, inserting the NA genes of clone 7a and A/Fiji into A/Victoria and the NA gene of A/Fiji into clone 7a had no affect on apoptosis, even though the three parent viruses have different NA activities based on equal number of particles (Table 2). At equal $TCID_{50}$, a virus sample contains an equal number of infectious particles but an unknown number of non-infectious particles, all with NA activity. NA induces apoptosis at the cell surface before virus enters the cell (Morris et al., 1999) so both infectious and non-infectious particles can contribute to the process. It can thus be argued that measuring the NA activity of the inoculum measures the NA activity relevant to the induction of apoptosis. Therefore, the NA activity of the inocula was determined. Even though NA activities of rVic7aNA and rVicFNA inocula were higher than that of rVictoria, the level of apoptosis induced by all three viruses was not significantly different. Similarly, the NA activity of the r7aFNA inoculum was considerably lower than that of clone 7a, yet the level of apoptosis induced was similar. In addition, clone 7a and A/Fiji had comparable NA activities at the inocula used, but the levels of apoptosis were significantly different. These results suggest that the role of NA in influenza virus-induced apoptosis is limited and not responsible for the differential induction of apoptosis observed between A/Victoria, clone 7a, and A/Fiji.

Neither HA nor M caused a significant difference in the induction of apoptosis. This was despite the fact that overexpression of HA and M from plasmids has been reported to induce apoptosis through endoplasmic reticulum stress (Flory et al., 2000) and that M1 may be a caspase inhibitor (Timofeeva et al., 2001).

To maintain the appropriate balance between attachment/entry and release/spread, the NA and HA evolve together; viruses with a strong affinity for their receptors require a highly active NA (Mitnaul et al., 2000). However, in this study, no differences in NA activity or binding affinity, based on equal number of particles, were observed for the recombinant viruses with exchanged NA or HA genes with the exception of r7aFNA (Table 2a). The NA activity of r7aFNA was higher than that of A/Fiji but still lower than that of clone 7a. However, sequence analysis of segment 6 from r7aFNA did not reveal any changes compared to that from A/Fiji. This difference in NA activity must therefore relate to number of NA per virion. Interestingly, clone 7a is a spherical virus (C. Sweet, unpublished observations), whereas A/Fiji is filamentous (W. Barclay, unpublished observations). A difference in shape may result in differences in number of NA per virion accounting for the difference in NA activity observed.

The single gene exchange recombinant viruses suggest that NS is involved in influenza virus-induced apoptosis whereas NA, HA, and M are not. However, inserting the M, NA, and HA gene of clone 7a into A/Fiji and vice versa confounded this latter conclusion. rFiji7aM/NA/HA, which retains the A/Fiji NS gene, induced significantly more apoptosis than A/Fiji (Table 2c). Similarly, r7aFM/NA/HA induced significantly less apoptosis than clone 7a (Table 2c). Together, M, NA, and HA have an effect on apoptosis that they did not exert individually. Inserting clone 7a M, NA, and HA into A/Fiji induced a less dramatic effect compared to inserting A/Fiji M, NA, and HA into clone 7a, probably due to the inhibitory effect exerted by A/Fiji NS in rFiji7aM/NA/HA. These differences may also be due to a difference in virion shape. However, a change in virion shape from spherical to filamentous or vice versa cannot be solely responsible for these results as rVic7aM and rFiji7aM, both of which would be predicted to be spherical, did not induce a significant difference in apoptosis compared to rVictoria and A/Fiji, respectively, both of which are filamentous (Elleman and Barclay, 2004; W. Barclay, unpublished observations). However, it has been

a.

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                1
clone 7a NS1    MDSNTVSSFQ VDCFLWHVRK QVVDQELGDA PFLDRLRRDQ KSLRGRGSTL
Fiji NS1        MDPNTVSSFQ VDCFLWHVRK QVADQELGDA PFLDRLRRDQ KSLKGRGSTL
Victoria NS1    MDSNTVSSFQ VDCFLWHVRK QIVDQELGDA PFLDRLRRDQ KSLRGRGSTL
                  †                   †                     †
                  *                  **                     *

               51
clone 7a NS1    GLNIEAATRV GKQIVERILK EESDEALRMT MASAPASRYL TDMTIEELSR
Fiji NS1        GLDIETATCV GKQIVERILK EESDEALKMT MASAPASRYL TDMTIEEMSR
Victoria NS1    GLDIEAATHV GKQIVEKILK EESDEALTMT MASTPASRYI TDMTTEELSR
                  † †  †                   †                     †
                     *  *                  *      *              *

               101
clone 7a NS1    DWFMLMPKQK VEGPLCIRID QAIMDKNIML KANFSVIFDR LETLILLRAF
Fiji NS1        DWFMLMPKQK VAGPLCVRMD QAIMDKNIIL KANFSVIFNR LETLILLRAF
Victoria NS1    DWFMLMPKQK VEGPLCIRID QAIMDKNIML KANFSVIFDR LETLILLRAF
                           †    † †          †          †
                           *    * *          *          *

               151
clone 7a NS1    TEEEAIVGEI SPLPSLPGHT IEDVKNAIGV LIGGLEWNDN TVRVSKNLQR
Fiji NS1        TEEGAIVGEI SPLPSLPGHT NEDVKNAIGV LIGGLEWNDN TVRVSKTLQR
Victoria NS1    TEEGAIVGEI SPLPSFPGHT IEDVKNAIGV LIGGLEWNDN TVRVSKTLQR
                   †                  †                         †
                              *     *


               201
clone 7a NS1    FAWRSSNENG RHPLTPKQKR
Fiji NS1        FAWRSSNENG RPPLTPKQKR KMAGTIRSEV RRNKMAD
Victoria NS1    FAWGSSNENG GPPLTPKQKR KMARTARSKV RRDKMAD
                          †
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b.

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                1                                                   50
clone 7a NS2    MDSNTVSSFQ DILLRMSKMR LGSSSEDLNG MITQFESLKI YRDSLGEAVM
Fiji NS2        MDPNTVSSFQ DILMRMSKMQ LGSSSGDLNG MITQFESLKL YRDSLGEAVM
Victoria NS2    MDSNTVSSFQ DILLRMSKMQ LGSSSEDLNG MITQFESLKL YRDSLGEAVM
                  †           †     †     †              †
                  *           *           *

               51                                                  100
clone 7a NS2    RMGDIHSLQN RNGKWREQLG QKFEEIRWLI EEVRHRLKIT ENSFEQITFM
Fiji NS2        RMGDLHSLQN RNGKWREQLG QKFEEIRWLI EEVRQKLKIT ENSFEQITFM
Victoria NS2    RMGDLHLLQN RNGKWREQLG QKFEEIRWLI EEVRHRLKTT ENSFEQITFM
                   †                              ††
                      *                           **  *

               101
clone 7a NS2    QALQLLFEVE QEIRTFSFQL I
Fiji NS2        QALQLLFEVE QEIRTFSFQL I
Victoria NS2    QALQLLFEVE QEIRTFSFQL I
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Fig. 6. Amino acid alignments of (a) NS1 and (b) NS2 for clone 7a, A/Fiji, and A/Victoria. Amino acid differences between A/Fiji and A/Vic are indicated with an asterisk (*) whereas amino acid differences between A/Fiji and clone 7a are indicated with a cross (†).

reported that changes in HA and NA cytoplasmic tails alone can result in irregularly shaped virions (Jin et al., 1997; Mitnaul et al., 1996). In addition, mutational analysis of the cytoplasmic tail and transmembrane domain of NA has revealed that NA can influence virus shape, size, and titer (Barman et al., 2004), thus it cannot be ruled out that changing all three (NA, HA, and M) genes would have a more profound effect. The level of apoptosis induction could be related to differences in viral infection of morphologically different viruses as differences in infection

were observed even though all experiments were performed at equal $TCID_{50}$/cell. However, this seems unlikely as total apoptotic cell death was calculated as a percentage of the total number of infected cells. Alternatively, the results may be due to differences in transcription of M, NA, and HA as the presence of a C or U at position 4 of the 3' terminus has been shown to be responsible for differences in transcription (Lee and Seong, 1998). However, the clone 7a and A/Fiji M, NA, and HA gene segments generated possess identical 3' termini (with a U at position 4) and thus should be expressed at comparable levels.

Inserting A/Fiji NP into A/Victoria induced a significant increase in the induction of apoptosis compared to rVictoria (Fig. 2). In contrast, inserting A/Fiji NP into clone 7a resulted in a significant decrease in the level of apoptosis compared to clone 7a (Fig. 2). These results suggest that the effect of A/Fiji NP is dependent on its interaction with another viral protein and the degree of interaction is different between the A/Victoria and clone 7a viruses. The primary role of NP is to encapsidate the virus genome but it is multifunctional. It interacts with many cellular and viral proteins and is thus a key adaptor molecule between viral and host processes (Portela and Digard, 2002). NP interacts directly with PB1 and PB2 but not PA (Biswas et al., 1998) and in addition binds M1 (Ye et al., 1999). Interestingly, at the amino acid level, A/Fiji NP is 97.8% and 94.8% identical to that from A/Victoria and clone 7a, respectively, whereas clone 7a NP shares 97% identity with A/Victoria NP. There are two (N34D and R77K) and four (K47R, S50N, N34G, and R77K) amino acid changes located in the minimal RNA binding region (Portela and Digard, 2002) of A/Fiji NP compared to A/Victoria and clone 7a, respectively. These changes also locate within the PB2 binding region. A/Fiji contains further a 6 and 16 amino acid differences compared to A/Victoria and clone 7a, respectively, in this region. In addition, changes were observed in the C-terminal region responsible for the suppression of PB2-NP binding, and in the oligomerization regions. It is unknown at present how these sequence differences relate to the differential induction of apoptosis observed.

The role of the polymerase proteins, per se, in the induction of apoptosis has not previously been studied. Inserting A/Fiji PB2 into A/Victoria or clone 7a resulted in a decrease in apoptosis compared to the respective parent viruses (Fig. 4), which again could be due to a change in the degree of compatibility with parent NP. However, the level of apoptosis induced by rVicFpoly, containing A/FijiPB2 but not NP, did not induce a significantly different level of apoptosis compared to rVictoria (Fig. 5). In contrast, rVic7apoly induced a significant increase in apoptosis induction compared to rVictoria (Fig. 5), even though no effect was observed when clone 7a PB2 alone was inserted into A/Victoria (Fig. 4). PB2 interacts with RNA, NP, and PB1. Mutations in PB2, along with PB1 and NA, have been shown to correlate with the induction of apoptosis in lymphocytes by H5N1 influenza viruses (Katz et al., 2000; Tumpey et al., 2000), although whether this was due to viral replication in lymphocytes or the indirect action of cytokine induction was not evident.

The PA gene of clone 7a originates from A/PR/8/34, a H1N1 virus like A/Fiji. However, sequence analysis reveals that the A/Fiji PA gene shares more identity with A/Victoria than clone 7a, which is reflected in the effect of exchanging this gene. Inserting clone 7a PA gene into A/Victoria resulted in a significant increase in apoptosis (Fig. 3). In contrast, inserting A/Fiji PA gene into clone 7a caused a reduction in the level of apoptosis induced. Therefore, the effect of A/Fiji PA is, like A/Fiji NP, dependent upon the genetic background into which it is inserted, indicating that PA interacts via another viral or cellular protein. Further analysis of these viruses showed that exchanging the PA gene may affect the rate of progression of apoptosis, as more cells were morphologically apoptotic at 12 h for both rVic7aPA and rVicFPA compared to rVictoria (data not shown). PA has been shown to induce generalized proteolysis leading to a decrease in the steady state of co-expressed proteins (Sanz-Ezquerro et al., 1996). This action could potentiate the apoptotic response, which is propagated via a proteolytic cleavage cascade. The dependence on genetic background would then suggest that this action is modulated by the virus.

Inserting clone 7a PB1 into A/Victoria had no effect on the level of apoptosis induced compared to rVictoria whereas inserting A/Fiji PB1 into clone 7a significantly lowered the level of apoptosis. PB1 is the main catalytic component of the polymerase complex, possessing both transcriptase and replicase activity. Therefore, exchanging the PB1 gene may have a profound effect on these processes. However, segment 2 encoding PB1 also encodes for another protein, PB1-f2. It is unknown which of these proteins is affecting apoptosis. PB1-f2 induces apoptosis in vitro, in a cell-specific manner (Chen et al., 2001), by the formation of ion channels in the mitochondrial membrane (Chanturiya et al., 2004). A/Fiji segment 2 encodes a truncated PB1-f1 of 57 amino acids compared to the 88 amino acid protein encoded by clone 7a. A/Fiji is not unique in encoding a truncated PB1-f2 as recent isolates from Taiwan have also been shown to encode a 57 amino acid PB1-f2 (Chen et al., 2004). The mitochondrial localization signal is located within the C-terminal end of PB1-f2, therefore translocation of A/Fiji PB1-f2 to the mitochondria will not occur. This may account for the reduction in apoptosis observed when A/Fiji PB1 was inserted into clone 7a. However, Chen and colleagues reported that the apoptosis inducing ability of PB1-f2 was restricted to non-permissive cells (Chen et al., 2001).

Although caspase 3 has been shown to be essential for virus replication in MDCK cells (Wurzer et al., 2003), the results obtained during this study for the parent viruses, clone 7a, rVictoria, and A/Fiji, and their recombinants indicated no relationship between apoptosis and virus yields

(Figs. 2–5). In support of the results presented here, Brydon and colleagues recently reported that caspase inhibitors did not affect virus yields generated in human bronchiolar cells (Brydon et al., 2003). In addition, Mersich et al. (2004) have recently shown no relationship exists between yields and apoptosis induced by circulating influenza viruses in Argentina and some influenza viruses that induced low levels of apoptosis replicated to high titers in a porcine cell line (Seo et al., 2001).

It is difficult to attribute the induction of apoptosis to a single viral protein from these results. A/Fiji NS1 and/or NEP down-regulates apoptosis more efficiently than that from clone 7a or A/Victoria. However, by changing the M, NA, and HA genes, the effect of NS1 on apoptosis can be reduced. The level of apoptosis induced appears to depend on the balance between the pro- and anti-apoptotic functions of several viral proteins. These results indicate that it is hard to predict the properties of newly emerging influenza virus strains based on those of the parents. This also applies to other aspects of virulence, which is multigenic, and would make it difficult to judge the potential virulence of new isolates such as the 1997 and 2003/2004 H5N1 Asian isolates (Guan et al., 2004). Some of these isolates were much more virulent than others and the genetic basis for this is unclear. Data obtained during this study indicate that both the background virus in which the gene being is inserted as well as the gene exchanged must be considered in these situations. In this respect, it is interesting that the insertion of the 1918 virus HA into several influenza backgrounds increased their virulence, but this was dependent on the genetic background of the virus; the enhanced virulence of the viruses was reduced if the 1918 NA was inserted together with the HA (Kobasa et al., 2004). Further studies of other aspects of pathogenicity using the approaches described here are necessary to increase our understanding of the epidemic potential of newly emerged viruses.

Materials and methods

Biological materials

MDCK (NBL-2) and 293T cells were maintained in growth medium [Dulbecco's modified Eagle medium (DMEM) with pyridoxine supplemented with 1× essential amino acids, 10% fetal calf serum, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 g/ml streptomycin (Invitrogen)]. The virus strains A/Fiji/15899/83 (H1N1) (A/Fiji) and clone 7a (H3N2) of the A/Puerto Rico/8/34 (H1N1) × A/England/939/69 (H3N2) reassortant system and their growth have been described previously (Price et al., 1997). All recombinant viruses were passaged in MDCK NBL-2 cells in serum-free medium containing 1 g/ml TPCK-treated trypsin. All virus titers were determined by titrating in MDCK (NBL-2) cells in 96-well plates. After 48 h, the supernatants were assayed for hemagglutination to determine which well monolayers were infected and viral titers expressed as log10$TCID_{50}$/ml, calculated using the method of moving averages (Thompson, 1947). Plasmids containing cDNA copies of the vRNA segments from A/Victoria/3/75 (A/Victoria) cloned into the transcription plasmid pPolIRT (Jackson et al., 2002), and the vector pPolIRT*Bsm*BI, were kindly provided by Dr. Thomas Zuercher (GlaxoSmithKline, Stevenage, UK). The transcription plasmid pPolI*Sap*IRib and the expression plasmids, pGT-h-PB1, pGT-h-NP, pGT-h-PB2, and pGT-h-PA, encoding A/NWS/33 PB1 and NP and A/PR/8/34 PB2 and PA, respectively (Fodor et al., 1999), were kindly provided by Prof. George Brownlee (Oxford University, Oxford, UK).

Plasmid construction

The pPolI-derived transcription plasmids, pPolFNS, pPolFM, pPolFNA, pPolFNP, pPolFHA, pPolFPA, pPolFPB1, pPolFPB2 containing cDNA copies of the 8 viral RNA segments from A/Fiji and pPol7aNS, pPol7aM, pPol7aNA, pPol7aNP, pPol7aHA, pPol7aPA, pPol7aPB1, pPol7aPB2 containing cDNA copies of the 8 viral RNA segments from clone 7a were generated as follows. RNA was extracted from virus stocks and cDNA generated as described previously (Morris et al., 2002). The cDNAs were then amplified using the EXPAND PCR system (Roche). Primers, specific for each segment, which inserted either a *Sap*I or *Bsm*BI restriction site at the termini of the 5′ and 3′ untranslated regions were used (sequences available on request). All primers were designed to contain a U at position 4 of the 3′ terminus. The PCR products were then cloned into the blunt TOPO cloning vector, pCR4 (Invitrogen). The inserted segment was fully sequenced, to check for PCR-generated mutations, using the version 3.0 Big Dye Terminator ready reaction cycle sequencing kit (Applied Biosystems). Inserts of correct sequence (100% identity to the virus segment at the amino acid level) were subcloned into either pPolI*Sap*IRib or pPolIRT*Bsm*BI. The inserted DNA was sequenced over the cloning junction, as described above, to confirm the orientation of the cloned DNA. All sequence analysis was carried out using the influenza virus sequence database (ISD; www.Flu.lanl.gov; Macken et al., 2001).

Virus rescue

To rescue infectious virus, 293T cells were seeded at a density of 1×10^5 cells/well in a 12-well plate. Cells were transfected with 8 transcription plasmids (generally seven plasmids derived from one virus and 1 plasmid derived from another virus) (0.5 μg each plasmid) and 4 expression plasmids (0.5 μg pGT-h-PB1, pGT-h-PB2, and pGT-h-PA and 1 μg of pGT-h-NP) prepared using the Plasmid Miniprep kit (Qiagen). Transfections were performed using Fugene6 (Roche) at a ratio of 1:3 in growth medium with serum reduced to 3%. After 24 h, the transfected 293T cells were co-

cultured with MDCK (NBL-2) cells as described previously (Elleman and Barclay, 2004). Cells were examined for cytopathic effect (cpe) over 4–5 days; supernatants were harvested after complete cpe was observed. Cell debris was pelleted by centrifugation at 4000 rpm for 10 min at 4 °C. The presence of virus in the supernatant was confirmed by hemagglutination assay. The genotype of each recombinant virus was confirmed by sequencing. Viral RNA was extracted as previously described (Morris et al., 2002) and the exchanged RNA segment and a background RNA segment, chosen at random, were amplified by RT-PCR and sequenced as described above.

Determination of the percentage of apoptotic and infected cells

5×10^4 MDCK (NBL-2) cells were infected at an moi of 4 or 2$TCID_{50}$/cell, depending on the maximum titer achieved by each virus, as described by Price et al. (1997). Cytotoxicity assays were performed using the Cytotox96 assay (Promega). Morphological apoptosis was determined by nuclear staining with propidium iodide (Price et al., 1997). Infected cells were identified using monoclonal antibody to the nucleoprotein of the X-31 (H3N2) influenza virus strain and anti-mouse-FITC conjugate as previously (Price et al., 1997). Total apoptotic cell death, determined from percentage cytotoxicity (which measures the percentage of apoptotic cells which have entered late apoptosis, detached from the monolayer, and lysed due to the absence of phagocytes) and morphological apoptosis (which measures those cells remaining attached to the coverslip at 24 h pi), was calculated as described by Mohsin et al. (2002). Cytotoxicity resulting from necrosis is taken into account in the calculation by subtracting the level of cytotoxicity generated in mock-infected cells.

Yields

Medium from infected wells was harvested prior to staining for apoptosis and infection. Viral yields were determined by titrating in MDCK (NBL-2) cells as described above. Virus yields were calculated based on the total number of infected cells.

NA activity

NA activity of the viruses was determined based on equal numbers of particles and equal infectivity to give a value for absolute NA activity per virion or the relative NA activity of the inoculum (4 or 2 $TCID_{50}$/cell) used in the apoptosis studies, respectively. The modified WHO assay using fetuin as a substrate was performed as described previously (Morris et al., 1999). NA activities are expressed as μmol of *N*-acetyl neuraminic acid (NANA) released over the 18-h incubation period and were calculated from a standard curve.

Hemagglutination-inhibition (HI) assays

These were performed as described by Sweet et al. (1974) using 4HA of each virus, 1% human erythrocytes, and horse serum which contains α-macroglobulin rich in α2-6 galactose linkages (Ryan-Poirier and Kawaoka, 1993). The HI titers are expressed as the reciprocal of the highest dilution that still caused complete inhibition of hemagglutination.

Statistical analysis

Data were collated and analyzed for statistical significance by the Student's *t* test.

Acknowledgments

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